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J Immunol 1998; 160:4361-4366;
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The significance of the glycosylphosphatidylinositol (GPI) anchor is unknown. Since GPI-anchored proteins mediate signaling, it has been suggested that the GPI structure serves as a signal-transducing element. However, the division of signaling functions between transmembrane and GPI-anchored proteins is unclear. Studies of distinct membrane-anchored forms of the same protein may resolve this issue. The adhesion molecule CD58 is expressed on the cell surface in both a transmembrane and a GPI-anchored form and hence provides a useful model. We studied CD58 in the human B lymphoblastoid cell line JY. In addition to mediating adhesion, CD58 is involved in signal transduction. Incubation of JY cells with immobilized anti-CD58 Abs results in extensive tyrosine phosphorylation and in secretion of TNF-α. We demonstrate that CD58 is associated with protein kinases(s) and with several kinase substrates. We further demonstrate that both CD58 isoforms are involved. CD58 in JY variant cells, which express only the transmembrane form, as well as CD58 in JY variant cells, which express only the GPI-anchored form, are associated with kinase activity. This association results in a phosphorylation pattern that is common to the variant and to wild-type JY cells. Thus, these findings suggest that the capacity of GPI-anchored proteins to interact with kinases is not always dependent on the GPI anchor itself. The Journal of Immunology, 1998, 160: 4361–4366.

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ome cell surface proteins lack a transmembrane-spanning domain and are anchored to the outer leaflet of the cell membrane by a glycosylphosphatidylinositol (GPI) moiety (1–3). The physiologic significance of the novel GPI anchor is as yet unknown. GPI-anchored proteins can mediate signaling. In T cells, for example, cross-linking of any GPI-anchored protein causes a rise in intracellular calcium, cytokine production, and mitogenesis in the presence of phorbol esters (4, 5). This recurrent relationship between triggering capability and membrane attachment has led to the suggestion that the GPI anchor may be involved in signal transduction. Since GPI-anchored proteins are restricted to the outer leaflet of the membrane lipid bilayer and lack cytoplasmic domains, it is not clear how the activation signal is transmitted. GPI-anchored proteins associate with tyrosine kinases (6, 7). They colocalize in detergent-resistant complexes that contain cholesterol, glycolipids, protein tyrosine kinases, and some of their substrates (8–13). The existence of these large complexes in specialized membrane domains may explain the surprising phenomenon of signaling through GPI-anchored receptors and support a functional role for the GPI moiety as a signal transducing element. However, since many transmembrane proteins can associate with tyrosine kinases and mediate signaling, the division of the signaling function between transmembrane and GPI-anchored proteins is unclear. Comparative studies of distinct membrane-anchored forms of the same polypeptide may resolve this issue.

The adhesion molecule CD58 (LFA-3) plays an important role in immunoregulation. In addition to mediating intercellular adhesion by binding to its ligand, CD2 (14–16), it is also involved in signal transduction (17–19). CD58 is expressed on the cell surface of nucleated cells in both a transmembrane and a GPI-anchored form (20–22) and hence provides a model for the study of signaling by two distinct membrane-anchored forms of the same protein. In this report, we demonstrate that both the transmembrane form and the GPI-anchored form of CD58 can associate with protein kinases. Thus, the mere capacity of a particular protein to interact with kinases is not necessarily directed by the type of membrane anchor.

Materials and Methods

Cells

The human B lymphoblastoid cell line JY and the variant cells derived from it were maintained in RPMI 1640 medium supplemented with 10% FCS. The mouse L929 cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS.

Antibodies

The hybridoma TS2/9 secreting mouse anti-human CD58 (23), the hybridoma TS2/18 secreting mouse anti-human CD2 (23), and the hybridoma W6/32 secreting mouse anti-HLA class I (24), were purchased from the American Type Culture Collection (Rockville, MD). The hybridoma 145-2C11 secreting hamster anti-murine CD3 ε (25) was kindly provided by Dr. J. A. Bluestone (University of Chicago, Chicago, IL). mAbs secreted by the hybridoma cell lines were purified from ascites by protein G-Sepharose (Pharmacia, Uppsala, Sweden). The biotin-conjugated anti-phosphotyrosine Ab 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). F(ab)’2 of goat anti-human Ig was obtained from Zymed (South San Francisco, CA). Anti-ζ Abs were raised in rabbits immunized with the synthetic peptide RRRGKGHDGLYQG from the carboxyl-terminal region of the protein.
Reagents
Detergents, protease inhibitors, and phosphatase inhibitors were purchased from Sigma (St. Louis, MO). \([\gamma-32P]ATP\) and \([32P]orthophosphate\) were purchased from Amersham (Buckinghamshire, England). Recombinant human TNF-\(\alpha\) and \(N\)-glycanase were purchased from Genzyme (Cambridge, MA). Horseradish peroxidase (HRP)-conjugated streptavidin was obtained from Jackson ImmunoResearch (West Grove, PA).

Ab cross-linking of CD58
Flat-bottom 24-well culture plates were incubated with 10 \(\mu\)g/ml of TS2/9 mAb in PBS for 20 h at 4°C. The plates were rinsed with PBS, incubated for 1 h at 37°C with PBS containing 1% BSA, and rinsed with medium. JY cells were incubated in Ab-coated wells at 5 \(\times\) 10^5 cells/well in 1 ml of medium.

Assay for TNF-\(\alpha\)
Culture supernatants of JY cells incubated for 24 h with immobilized Abs were assayed for TNF-\(\alpha\) activity as previously described (26). Briefly, L929 cells were plated at 2.5 \(\times\) 10^5 cells/well in flat-bottom 96-well microplates. After 24 h, culture supernatants or rTNF-\(\alpha\) were added at different dilutions to the L929 monolayers in the presence of 1.5 \(\mu\)g/ml actinomycin D. Plates were incubated for an additional 24 h and then fixed and stained with Hemacolor (26). SDS (0.5%) was added to each well, and the staining intensity was then determined by a microplate reader spectrophotometer at 630 nm. TNF-\(\alpha\) concentration in culture supernatants was extrapolated from standard curves with rTNF-\(\alpha\).

Cell biotinylation
For surface labeling with biotin, washed cells were resuspended at 5 \(\times\) 10^5 cells/ml in PBS, pH 8.0, containing 0.1 mg/ml sulfosuccinimidobiotin (Pierce Chemical Co., Rockford, IL). After 30 min at 4°C, cells were washed three times.

Cell radiolabeling
Cells were washed three times in phosphate-free RPMI 1640 and resuspended in this medium supplemented with 10% dialyzed FCS at a cell density of 4 \(\times\) 10^6/ml. [32P]Orthophosphate (0.4 mCi/ml) was then added, and cells were incubated for 2 h. Labeled cells were stimulated for 30 min as described below, then washed in PBS containing phosphatase inhibitors (400 \(\mu\)M sodium orthovanadate, 5 mM EDTA, and 10 mM sodium pyrophosphate). Washed cells were then solubilized.

Cell lysis, immunoprecipitation, kinase assay, and electrophoresis
Cells were solubilized in 1% Nonident P-40, except where described differently, for 30 min at 4°C. The lysis buffer contained 150 mM NaCl, 25 mM Tris-HCl, pH 8.0, 1 mM PMSF, 10 mM iodoacetamide, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate. Lysates were cleared by centrifugation at 12,000 \(\times\) g for 15 min at 4°C. CD58 or HLA were immunoprecipitated from cell lysates by the addition of 50 \(\mu\)l slurry of TS2/9-Sepharose or W6/32-Sepharose, respectively, and incubated for 2 h at 4°C with constant shaking. TCR components were immunoprecipitated by rabbit anti-\(\zeta\) Abs preadsorbed to protein A-Sepharose. Sepharose beads were then washed three times in lysis buffer and once in kinase buffer containing 100 mM NaCl, 50 mM HEPES, pH 7.3, and 5 mM MgCl2. The beads were then resuspended in 50 \(\mu\)l of kinase buffer, 20 \(\mu\)Ci of \([\gamma-32P]ATP\) were added, and the kinase reaction was conducted at 4°C for 15 min. The reaction was stopped by washing three times with lysis buffer containing 25 mM EDTA. The immun complex was eluted from the beads by boiling in Laemmli sample buffer, and proteins were resolved by SDS-PAGE. Labeled proteins were detected by autoradiography. For alkaline hydrolysis of proteins, gels were treated with 1 M KOH for 2 h at 55°C as described (27).

Western blot analysis
For determination of tyrosine phosphorylation, lysate samples (30 \(\mu\)g protein) were subjected to SDS-PAGE and transferred to nitrocellulose. Membranes were probed sequentially with biotin-conjugated antiphosphotyrosine Abs and with HRP-conjugated streptavidin. The probed proteins were visualized by enhanced chemiluminescence. For detection of biotinylated cell surface CD58, immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose. Proteins were probed with HRP-conjugated streptavidin and visualized by enhanced chemiluminescence.

N-Glycanase treatment
CD58 eluted from Sepharose beads was incubated for 20 h with N-glycanase according to the supplier’s instructions (Genzyme). The proteins were precipitated with acetone in the presence of 10 \(\mu\)g tRNA as carrier and were dissolved in sample buffer.

Purification of CD58
CD58 was purified from Triton X-100 cell lysates by immunoadfinity chromatography on TS29-Sepharose as described (16). After intensive washing of the immunoadfinity column, CD58 was eluted with 50 mM glycine HCl, pH 3.0, 0.15 M NaCl, 1% octyl \(\beta\)-glucoside. Eluted fractions were immediately neutralized with 1 M Tris-HCl, pH 9.0, and analyzed by SDS-PAGE and silver staining. Selected fractions were pooled and passed through Centricron-30 filters (Amicon, Danvers, MA) for concentration and removal of detergent. Ultrafiltration was repeated three times, adding 2 ml of PBS and reducing the volume to 100 \(\mu\)l with each cycle.

Incorporation of CD58
Cells were washed three times, resuspended at 10 \(\times\) 10^6 cells/ml, and incubated at 37°C for 1 h with 10 \(\mu\)g/ml purified CD58. Cells were then washed three times.

Results
It has been previously reported that CD58 can transmit the necessary signals for the release of cytokines by human monocytes (18). To determine whether CD58 can also transmit signals in JY cells (B lymphoblastoid cells), immobilized mAb was used to cross-link surface CD58. Culture supernatants of JY cells, incubated for 24 h in plastic plates coated with TS2/9, were assessed for TNF-\(\alpha\) activity. JY cells constitutively release low amounts of TNF-\(\alpha\). However, a three- to fivefold increase over baseline production of TNF-\(\alpha\) was observed following CD58 cross-linking (Fig. 1). The up-regulation of TNF-\(\alpha\) release by JY cells was not caused by endotoxin contaminating TS2/9 preparations. Thus, incubation of JY cells for 24 h with concentrations of LPS reaching 10 \(\mu\)g/ml did not trigger release of TNF-\(\alpha\) (data not shown). Polymyxin B, which is an inhibitor of endotoxin activity, had no effect on the up-regulation of TNF-\(\alpha\) release by JY cells (Fig. 1A). In addition, immobilized control Abs such as anti-CD2, which were purified in a sequence with TS2/9 by the same protein G-Sepharose column, did not trigger TNF-\(\alpha\) release (Fig. 1B).

The activation of JY cells by anti-CD58 Abs involves tyrosine phosphorylation. As shown in Figure 2, incubation of JY cells with

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**FIGURE 1.** Up-regulated release of TNF-\(\alpha\) from JY cells following cross-linking of CD58. A, JY cells were incubated for 24 h on uncoated (medium) plates or on TS2/9 (anti-CD58)-coated plates in the absence or presence of polymyxin B (5 \(\mu\)g/ml). B, JY cells were incubated for 24 h on uncoated (medium) plates, TS2/9 (anti-CD58)-coated plates, or TS2/18 (anti-CD2)-coated plates. Culture supernatants were then tested for TNF-\(\alpha\) activity (see Materials and Methods).
immobilized TS2/9 resulted in extensive protein tyrosine phosphorylation. The pattern of phosphorylated proteins was different from the pattern observed in JY cells activated by anti-Ig Abs. We further examined whether CD58 is associated with kinase activity. CD58 was immunoprecipitated from lysates of nonactivated (untreated) JY cells. Kinase reactions performed on CD58 immunoprecipitates resulted in phosphorylation of several proteins (Fig. 3). Such an activity did not coimmunoprecipitate with several other cell surface proteins such as HLA class I. The kinase activity associated with CD58 persisted in different detergents, including Nonidet P-40, digitonin, and CHAPS (Fig. 3A). Therefore, in subsequent experiments we used Nonidet P-40 cell lysates.

To identify the type of in vitro kinase activity, gels containing the phosphoproteins were treated with alkali. Comparing labeled proteins before and after alkaline hydrolysis takes advantage of the fact that phosphotyrosine is relatively stable to base (27). We demonstrated a similar phosphoprotein pattern before and after alkali treatment (Fig. 3B), indicating that the kinase activity associated with CD58 was predominantly tyrosine kinase activity. To ascertain the efficacy of alkali treatment, we used as a positive control the phosphorylation of TCR components (Fig. 3). Such an activity did not coimmunoprecipitate with several other cell surface proteins such as HLA class I. The kinase activity associated with CD58 persisted in different detergents, including Nonidet P-40, digitonin, and CHAPS (Fig. 3A).

FIGURE 2. Phosphorylation of proteins on tyrosine following cross-linking of CD58. JY cells were incubated for 30 min in the absence (lane 1) or presence of immobilized TS2/9 (lane 2). F(ab')2 fragments of anti-human Ig (lane 3), or immobilized TS2/18 (lane 4). Cells were then lysed. Cell lysates were analyzed for tyrosine phosphorylated proteins by SDS-PAGE and Western blot analysis with anti-phosphotyrosine Abs. Molecular weight standards are indicated in kilodaltons.

FIGURE 3. coprecipitation of kinase activity with CD58. A, JY cells were lysed in lysis buffers containing 1% CHAPS (lanes 1 and 4), 1% digitonin (lanes 2 and 5), or 1% Nonidet P-40 (lanes 3 and 6). Postnuclear supernatants were immunoprecipitated with Abs to CD58 (lanes 1–3) or to HLA class I (lanes 4–6). The immunoprecipitated complexes were subjected to an in vitro kinase assay. They were then eluted from Sepharose beads, separated by SDS-PAGE, and analyzed by autoradiography. B, In vitro kinase assay was performed on CD58 immunoprecipitates from Nonidet P-40 JY cell lysates. Following protein separation by SDS-PAGE, the gel was treated with 1 M KOH for 2 h at 55°C. Autoradiograms taken before (lane 1) and after (lane 2) alkali treatment are shown. C, Murine splenocytes were labeled with [32P]orthophosphate and activated for 30 min with medium alone (lanes 1 and 5), 1:10 culture supernatant of 145-2C11 mAb (lanes 2 and 6), 2.5 μg/ml Con A (lanes 3 and 7), or 100 ng/ml PMA (lanes 4 and 8). Following cell lysis, immunoprecipitation with anti-ζ Abs, and protein separation by SDS-PAGE, the gel was treated with 1 M KOH for 2 h at 55°C. Autoradiograms taken before (lanes 1–4) and after (lanes 5–8) alkali treatment are shown.

We further considered which of the CD58 isoforms (the transmembrane or the GPI-anchored form) is associated with kinase activity. We have previously reported the isolation of JY variant cells, which lack expression of GPI-anchored proteins (29). These variant cells are deficient in PIG-A, a component in the early steps of GPI anchor biosynthesis (30). Therefore, they express only the transmembrane form of CD58 (29). The ability of CD58 from two variant clones (clone 5 and clone 33) to associate with kinase was compared with that manifested by clone 25 (a wild-type clone derived from the JY cell line) and by parental JY cells. Figure 4 demonstrates a similar association of CD58 with kinase activity and with a set of kinase substrates in wild-type and in GPI-deficient JY cells. Different autoradiogram intensities were examined in each experiment to verify the identity of phosphorylation patterns in wild-type and variant cells. These results indicate that transmembrane CD58 associates with protein kinase activity. Moreover, transmembrane CD58 can mediate signaling. As shown in Figure 5, incubation of GPI-deficient JY cells (clone 5) with immobilized anti-CD58 Abs resulted in up-regulated TNF-α release.

The interpretation of these results may imply either that the GPI-anchored CD58 is not associated with kinase activity or that the two isoforms are indistinguishable in this regard. A more conclusive interpretation may be provided by kinase assays in cells that express only the GPI-anchored CD58. Despite extensive efforts, we were unable to select a stable variant that expressed only...
TNF-α. Following cross-linking of CD58, variant clone 5 cells were incubated for 4 h and lysed. CD58 was immunoprecipitated, treated with N-glycanase, subjected to SDS-PAGE, and analyzed by Western blot analysis with HRP-conjugated streptavidin. Arrowheads indicate bands corresponding to transmembrane (TM) CD58 and GPI-anchored CD58. GPI-deficient cells (lane 1), CD58-deficient cells (lane 2), CD58-reconstituted cells (lane 3), and wild-type cells (lane 4) are presented.

Discussed

In this report, we demonstrate that the transmembrane form and the GPI-anchored form of the adhesion molecule CD58 are associated with kinase activity. Incubation of JY cells with immobilized anti-CD58 mAbs resulted in extensive tyrosine phosphorylation and up-regulation of the release of TNF-α. Up-regulation of TNF-α release by JY cells is similarly mediated by cross-linking of HLA class II Ags (34). JY cells express the 75-kDa TNF-α receptor. It has been demonstrated that exogenous as well as endogenous TNF-α mediated the enhancement of NF-κB-like activity through the binding of the TNF-α receptor (34). Thus, the secreted TNF-α may function as an autocrine regulator of cell activation.

The GPI anchor has generally been considered required for cell activation. Studies with decay accelerating factor (DAF or CD55), Ly-6, and Qa-2 suggested a critical role for the GPI anchor in signal transduction mediated by these molecules (35–37). However, this hypothesis has recently become controversial, since it has been reported that the transmembrane form of CD73 can deliver signals (38). This contradiction may be accounted for by the procedures used for preparation of the transmembrane fusion constructs. For instance, 43 COOH-terminal amino acids of the GPI-anchored CD55 were removed to incorporate the membrane and cytoplasmic domains of a transmembrane protein (37). This change in the COOH-terminal region of CD55 could have altered

FIGURE 4. Coprecipitation of kinase activity with transmembrane CD58. GPI-deficient variant clone 5 (lane 1), GPI-deficient variant clone 33 (lane 2), wild-type clone 25 (lane 3), and the wild-type JY cell line (lane 4) were lysed in Nonidet P-40 lysis buffer. Postnuclear supernatants were immunoprecipitated with Abs to CD58. The immunoprecipitated complexes were subjected to an in vitro kinase assay. They were then eluted from Sepharose beads, separated by SDS-PAGE, and analyzed by autoradiography. A and B show different exposures of the same gel (1 day and 4 days, respectively).

FIGURE 5. Up-regulated release of TNF-α from GPI-deficient JY cells following cross-linking of CD58. Variant clone 5 cells were incubated for 24 h on uncoated (medium) plates, TS2/9 (anti-CD58)-coated plates, or TS2/18 (anti-CD2)-coated plates. Culture supernatants were then tested for TNF-α activity.

FIGURE 6. Expression of GPI-anchored CD58 following incorporation into JY cells. GPI-anchored CD58 was purified from human RBC. CD58-negative JY cells were incubated for 1 h with 10 μg/ml purified CD58, washed three times, and labeled with biotin. Control wild-type cells and GPI-deficient clone 5 cells were similarly labeled. Following biotinylation, cells were incubated for 4 h and lysed. CD58 was immunoprecipitated, treated with N-glycanase, subjected to SDS-PAGE, and analyzed by Western blot analysis with HRP-conjugated streptavidin. Arrowheads indicate bands corresponding to transmembrane (TM) CD58 and GPI-anchored CD58. GPI-deficient cells (lane 1), CD58-deficient cells (lane 2), CD58-reconstituted cells (lane 3), and wild-type cells (lane 4) are presented.
its signaling function independently of the membrane anchoring form. In addition, in the CD55, Ly-6, and Qa-2 systems, transmembrane forms were engineered to include the cytoplasmic portion of the respective transmembrane fusion proteins, whereas in the CD73 system the cytoplasmic portion of the transmembrane fusion protein was deleted (38). The advantage of the CD58 model is that the sequence of the two forms is identical throughout the entire extracellular domain from the amino terminus to the membrane attachment site (21, 22), and the cytoplasmic portion is expressed in its natural transmembrane form. Hence, it allows comparison of two distinct membrane-anchored forms of the same polypeptide. Our findings show that the transmembrane CD58 associates with kinase activity and mediates signaling. Thus, the signal transduction capacity of GPI-anchored proteins is not always dependent on the GPI anchor itself.

Since GPI-anchored proteins are restricted to the outer leaflet of the membrane lipid bilayer and lack cytoplasmic domains, it is not clear how they transmit activation signals. GPI-anchored proteins cluster in membrane microdomains, which are enriched in glycolipids and cholesterol (8–13). In these microdomains, the GPI-anchored proteins are complexed with src-like kinases, suggesting a mechanism by which these proteins mediate signaling (6, 7). However, since GPI-anchored proteins and src-like kinases are restricted to opposite leaflets of the bilayer, they cannot bind each other directly. A transmembrane linker protein that mediates the interaction between the two has been postulated. The GPI-anchored protein and the bridging molecule most probably interact via their exodomains. The transmembrane CD58 has a short cytoplasmic tail of 12 amino acids. It is conceivable, therefore, that the transmembrane CD58 also interacts with cytoplasmic signaling elements via a linker protein. Since the two CD58 isoforms have an identical extracellular sequence, they may interact with an identical linker protein and hence demonstrate similar association with kinases. Therefore, kinase association of a particular GPI-anchored protein may depend on its overall structure rather than on its possessing a GPI anchor. This suggestion is supported by the demonstration that a GPI-anchored form of CD4, constructed with the GPI anchor of CD58, lost the signaling activity of transmembrane CD4 (39), implying that membrane attachment by the CD58 GPI anchor is not sufficient to direct association with kinase activity.

We have previously demonstrated that the turnover characteristics and the adhesiveness potential of CD58 were not directed by the type of its membrane anchor (29, 40). As it is demonstrated here that association with signaling elements is independent of the type of membrane anchor, the properties conferred upon a protein by a GPI anchor remain elusive. Kinase complexes with GPI-anchored CD58, in contrast to complexes with transmembrane CD58, may be confined to the specialized membrane microdomains, in which GPI-anchored proteins cluster in large complexes. Division of membrane localization may be important for functional differences between the two protein isoforms. GPI-anchored and transmembrane molecules internalize through distinct pathways (41). The GPI-anchored proteins can be internalized via non-clathrin-coated invaginations within the glycolipid-enriched domains (13). It has been suggested that internalization is involved in signaling through GPI-anchored molecules (42). Alternatively, it has been suggested that complexed GPI-anchored proteins may be involved in activation-induced membrane vesiculation (43, 44).

FIGURE 7. Coimmunoprecipitation of kinase activity with GPI-anchored CD58. The following JY cells were used: wild-type cell line (lane 1), wild-type clone (lane 2), GPI-deficient clone expressing only transmembrane CD58 (lane 3), CD58-negative clone incorporated with GPI-anchored CD58 (lane 4), and CD58-negative clone (lane 5). In vitro kinase assay was performed on CD58 immunoprecipitates as described in the legend to Figure 4.

References


